**Apontic regulates cell proliferation and development by activating the expression of
hedgehog and *cyclin E***

Running title: Apt regulates *hh* and *cyclin E*

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Summary statement

We identified a novel role for Apontic as an important common regulator of the transcription of *hedgehog* and *cyclin E*. Our study provides important insights into the mechanism of organ development.

ABSTRACT

Hedgehog (Hh) signaling pathway and Cyclin E are key players in cell proliferation and development. Hyperactivation of *hh* and *cyclin E* has been linked to several types of cancer. However, transcriptional regulation of *hh* and *cyclin E* are not well understood. Here we show that an evolutionarily conserved transcription factor Apontic (Apt) is an activator of *hh* and *cyclin E* in *Drosophila*. Apt directly promotes the expression of *hh* and *cyclin E* through its binding site in the promoter regions of *hh* and *cyclin E* during wing development. This Apt-dependent proper expression of *hh* and *cyclin E* is required for cell proliferation and development of the wing. Apt-mediated expression of *hh* and *cyclin E* can direct proliferation of Hh-expressing cells and simultaneous growth, patterning and differentiation of Hh-recipient cells. The discovery of the coordinated expression of Hh and principal cell-cycle regulator Cyclin E by Apt implicates insight into the mechanism by which deregulated *hh* and *cyclin E* promotes tumor formation.

INTRODUCTION

Animal development requires the organ patterning and growth. How these two processes are coordinated remains unclear. The *Drosophila* wing is an excellent model to study the regulation of gene expression during the organ patterning and cell growth. The wing disc is a sac-like structure composed of disc proper (DP) cells and peripodial epithelium (PE). During larval development, wing disc DP and PE cells proliferate extensively and are patterned, finally give rise to the adult wing (Milner et al., 1984). The Hh and Cyclin E can contribute to patterning and growth of the wing disc during development (Neufeld et al., 1998; Tabata and Kornberg, 1994).

Hh pathway is one of the major signaling pathways that control animal development from *Drosophila* to humans, which has been implicated in stem cell maintenance, cell migration, axon guidance and tissue regeneration (Beachy et al., 2004; Charron et al., 2003; Clement et al., 2007; Hochman et al., 2006). In the *Drosophila* wing disc, Hh expresses in posterior (P) compartment cells and spreads into the anterior compartment where it activates target genes such as *engrailed* (*en*), *patched* (*ptc*), *collier* (*col*), *decapentaplegic* (*dpp*) and *iroquois* (*iro*) (Matusek et al., 2014; Nahmad and Stathopoulos, 2009; Tabata and Kornberg, 1994) to control wing patterning. Moreover, Hh is required for transient fusion between the PE and the DP sides during regeneration of wing discs (McClure and Schubiger, 2005). Therefore, the expression of *hh* is vital in the wing disc. In the anterior (A) compartment cells, the truncated transcription repressor Ci^R inhibits the transcription of *hh*. However, the underlying mechanism by which the posterior cells activate *hh* transcription is still to be determined.

Cyclin E belongs to the cyclin family, which is required for cell division (Knoblich et al., 1994). Dysregulation of *cyclin E* correlates with various tumors, including breast cancer and lung cancer (Donnellan and Chetty, 1999; Keyomarsi et al., 1994; Moroy and Geisen, 2004). Besides, deregulated Cyclin E activity causes cell lineage-specific abnormalities, such as impaired maturation due to unregulated cell proliferation (Minella et al., 2008). In *Drosophila*, Cyclin E is essential for G1-to-S phase transition in the posterior cells of eye disc (Richardson et al., 1995). It has been reported that *cyclin E* is a potential target gene of Hh signaling in *Drosophila*. Hh pathway activates *cyclin E* transcription through its unique transcription

90 factor Ci in the posterior cells of eye disc (Duman-Scheel et al., 2002). It is known that Hh
91 pathway is turned on exclusively in the A cells near A/P boundary (Strigini and Cohen, 1997;
92 Tabata and Kornberg, 1994). However, *cyclin E* expresses throughout the wing disc (Neufeld
93 et al., 1998). This contradiction suggests that other factors are involved in regulating the
94 expression of *cyclin E*. Therefore, it is fruitful to investigate the regulation of Cyclin E in
95 wing disc and the relationship between Cyclin E and Hh pathway.

96 Apontic (Apt) has been identified as a transcription factor involved in development of
97 tracheae, head, heart and nervous system (Eulenberg and Schuh, 1997; Gellon et al., 1997;
98 Liu et al., 2003; Su et al., 1999). Apt can suppress metastasis (Woodhouse et al., 2003) and is
99 required in the nervous system for normal sensitivity to ethanol sedation (McClure and
100 Heberlein, 2013). Moreover, Apt participates in JAK/STAT signaling pathway to limit border
101 cells migration (Starz-Gaiano et al., 2009; Starz-Gaiano et al., 2008; Yoon et al., 2011).
102 However, the role of Apt in wing development is unknown.

103 In this study, we found that both loss of and overexpression of *apt* resulted in defect wings.
104 Further studies demonstrated that loss of *apt* attenuated the expression of *hh* and *cyclin E*,
105 while *apt* overexpression upregulated *hh* and *cyclin E*. In addition, we found inherent Apt
106 binding sites in the promoter region of *hh* and *cyclin E*. Mutating the sites inhibited the
107 expression of *hh* and *cyclin E*. Collectively, Apt activates the expression of *hh* and *cyclin E* to
108 allow proper wing development.

109

110 **RESULTS**

111 **Apt is expressed in the wing disc and is required for wing development**

112 As the first attempt to investigate the function of *apt* during wing development, we analyzed
113 *apt* expression pattern in the wing disc by immunostaining using anti-Apt antibody. In the
114 wing disc, Apt was detected in PE cells as revealed by co-localization with a PE marker Ubx
115 (Fig. 1A). Apt was also detected in DP cells (Fig. 1B). These data clearly demonstrate that Apt
116 is expressed in both the PE and DP of the wing disc, suggesting its possible role in wing
117 development.

118 To analyze the role of Apt during wing development, we would examine the developing
119 wing of homozygous *apt* null mutant. However, *apt* null homozygotes die as embryos

(Eulenberg and Schuh, 1997). Therefore, we induced *apt* loss of function mutant clones in the wing disc using the *FLP/FRT* system (Theodosiou and Xu, 1998). The formation of these clones resulted in a small wing with a blistered phenotype (Fig. 1D) compared with the control wing (Fig. 1C). Furthermore, RNAi-mediated knockdown of *apt* in DP cells of the wing disc resulted in a small wing, and also reduced the width between vein 3 and vein 4 (Fig. 1E,F). Given that the space between vein 3 and vein 4 is a characteristic monitor of Hh pathway activity in adult wings, knockdown of *apt*-mediated narrowing the space indicates that Apt possibly regulates Hh pathway. RT-PCR analyses showed effective knockdown of *apt* mRNA level upon *apt*-RNAi (Fig. S1). To investigate the effect when *apt* is overexpressed, we employed *MS1096-Gal4* driver to express UAS-*apt* in both the PE and DP of the wing disc. Abnormal wings were induced by overexpression of *apt* (Fig. 1G). The wing was diminished and blistered, the pattern of veins was disrupted and extra abnormal bristles were induced in the wing margin. In addition, when UAS-*apt* was expressed by a stronger *gal4* (*sd-Gal4*) in DP cells, both wings and halteres were lost (Fig. 1H). Taken together, the loss-of-function and gain-of-function analyses indicate that Apt is indispensable for wing development.

Apt regulates the expression of *hh* in the wing disc

The observation that knockdown of *apt* narrowed the space between vein 3 and vein 4 implied that Apt might modulate Hh pathway in wings. As an important initiator of Hh pathway, *hh* gene expresses in the wing disc (Cho et al., 2000; Tabata and Kornberg, 1994). We first compared the expression of *apt* and *hh* in the wing disc, and found that Apt and *hh-lacZ* were co-expressed in PE cells (Fig. 2D-F) and posterior compartment cells of the DP (Fig. 2G-I) in the second instar larval disc. Furthermore, *apt* exhibited genetic interaction with *hh*. Transheterozygotes of two sets of *hh* alleles (*hh^{bar3}/hh²* and *hh^{Mir}/hh²*) exhibited smaller wing with an extra crossvein (Fig. S2), demonstrating that it is a phenotype of *hh* mutant. While wings of animals heterozygous for *hh²* or *apt*-null allele were normal, transheterozygotes of *apt*-null allele and *hh²* showed the same wing phenotype (Fig. 2A-C). These results raised the possibility that Apt regulates transcription of *hh*. To test the possibility, we analyzed the expression of *hh* under loss-of-function and overexpression of Apt. The expression of *hh-lacZ*

150 and Hh was significantly reduced in the *apt* mutant clones in the PE (Fig. 2J-L; Fig. S3A-C)
 151 and the DP (Fig. 2M-O; Fig. S3D-F). Moreover, the expression of *hh* was significantly
 152 reduced in the wing disc of larvae upon RNAi-knockdown of *apt* (Fig. S4). By contrast,
 153 overexpression of Apt increased the expression of *hh-lacZ* (Fig. 2P-R) and *hh* (Fig. S4,
 154 S5G-I). The expression of Hh also decreased in the *apt* mutant clones of the eye disc and the
 155 salivary gland (Fig. S5A-F). These results indicate that Apt activates the expression of *hh*.

156

157 **Apt directly controls *hh* in the wing disc**

158 To address how Apt activates the expression of *hh*, we focused on a 15-kb region of the *hh*
 159 locus known to reproduce the normal *hh* expression pattern in the wing disc (Lee et al., 1992).
 160 We identified one potential Apt binding sequence (Liu et al., 2003) within the region (Fig.
 161 3A). We next assessed the function of the Apt-binding site in *hh* using a CRISPR-Cas9
 162 system (Kondo and Ueda, 2013). Since the designed gRNA contained the Apt-binding site,
 163 four Apt-binding site deletion mutants and two insertion mutants were generated (Fig. 3B-C;
 164 Fig. S6A). Homozygotes of these mutations showed reduced expression of *hh* mRNA and Hh
 165 protein (Fig. 3D-E; Fig. S6B) and exhibited the small wing and reduced vein 3-4 spacing
 166 phenotypes (Fig. 3F; Fig. S6C-D). Effect of *hh*^{*AptDB1*} mutation on the *hh* function was also
 167 examined under the *hh*² heterozygous background. While wings of animals heterozygous for
 168 *hh*² or *hh*^{*AptDB1*} were normal, transheterozygotes of *hh*^{*AptDB1*} and *hh*² showed the same extra
 169 vein phenotype (Fig. 3G-I) as did transheterozygotes of *apt*-null allele and *hh*². Taken
 170 together, these data suggest that Apt directly regulates the expression of *hh* in the wing disc
 171 for proper wing development.

172

173 **Apt activates the *cyclin E* expression in the wing disc**

174 We have reported that Apt induces the *cyclin E* expression in the eye disc (Liu et al.,
 175 2014). Therefore, we examined whether Apt regulates *cyclin E* also in the wing disc. To do
 176 this, we first performed a double-staining experiment. In the wild-type wing disc, Apt and
 177 Cyclin E were co-expressed (Fig. 4A-C). Furthermore, the expression of Cyclin E was
 178 significantly reduced in the *apt* mutant clones (Fig. 4D-F; Fig. S7A). The expression of *cyclin*
 179 *E* mRNA was also reduced upon RNAi-knockdown of *apt* in the wing disc (Fig. S7B). By

contrast, the expression of Cyclin E and its mRNA was increased by overexpression of Apt in the wing disc using *MS1096-Gal4* and *UAS-apt* (Fig. 4G-I; Fig. S7B). These results indicate that Apt activates the expression of *cyclin E* in the wing disc.

We then asked whether the regulation of *cyclin E* by Apt is mediated through Hh. To test this idea, we compared the expression of *hh* and *cyclin E* upon overexpression of Apt in the wing disc. Both *hh* and Cyclin E were induced by overexpression of Apt (Fig. 2Q,4H). However, their expression patterns were different. Cyclin E was induced in all region of the wing disc, whereas the expression of *hh* was restricted in the posterior compartment. Moreover, the expression of Cyclin E was not changed by RNAi-knockdown of *hh* using *MS1096-Gal4* and *UAS-hh^{RNAi}* (Fig. 4J) and in an *hh* gain of function mutant *hh^{Mrt}* that exhibits ectopic expression of *hh* in the anterior compartment (Tabata and Kornberg, 1994) (Fig. 4K). These data suggest that the activation of *cyclin E* by Apt is independent of Hh in the wing disc (Fig. 4L).

Apt directly controls *cyclin E* in the wing disc

Since Apt directly activates the expression of *cyclin E* in the eye disc (Liu et al., 2014), we anticipated a direct role of Apt in the expression of *cyclin E* also in the wing disc. This expectation was verified by transgenic reporter assays. The reporter gene (Liu et al., 2014) carries the endogenous promoter and the *cyclin E* regulatory element containing a wild-type Apt-binding site (*cycEPlacZ*) (Fig. 5A) or a mutated site (*cycEMPLacZ*) (Fig. 5E). Although *cycEPlacZ* with the wild type binding site recapitulated the *cyclin E* expression in the wing disc (Fig. 5B-D), base substitutions in the Apt-binding site in *cycEMPLacZ* abolished the lacZ expression (Fig. 5F-H). These results indicate that Apt directly activates *cyclin E* through its binding site in the regulatory region of *cyclin E*.

Apt controls cell proliferation by inducing *hh* and *cyclin E*

Because both Hh and Cyclin E are involved in cell proliferation (Jiang and Hui, 2008; Knoblich et al., 1994), defects in *apt* would affect the cell number in the wing disc. As expected, we observed significant decrease in the cell number in an *apt* mutant clone using DAPI staining (Fig. 6B). Moreover, phalloidin labeling revealed disruption of the linear

arrangement of cells in the clone (Fig. 6C). When Apt was overexpressed in the wing disc, the number of DAPI-stained cells was not significantly changed from that in the control disc (compare Fig. 6F with 6D). However, the linear arrangement of cells was disrupted (compare Fig. 6G with 6E). Since Hh and Cyclin E are required for the regulation of apoptosis (Guerrero and Ruiz i Altaba, 2003; Hwang and Clurman, 2005; Ruiz i Altaba, 1999), we asked whether the overexpression phenotypes are caused by apoptosis. To test this, we investigated apoptosis in wing discs by staining with anti-Caspase-3 antibody. In the third instar wing disc from *apt* mutant clones and wild type, few apoptotic cells were observed (Fig. 6H-J). However, in the wing disc from an Apt-overexpressed larva, the number of apoptotic cells was significantly increased (Fig. 6K). This presumably explains why wing size was reduced upon overexpression of Apt (Fig. 1G).

Homozygotes of *hh* mutations for the Apt-binding site exhibited the small wing but not the blistered phenotype. However, *hh* and *cyclin E* double mutant recapitulates the smaller and blistered wing. While *CycE*^{2/+} flies showed normal wings, three percent of *hh*^{bar3}/*hh*^{bar3} and eighteen percent of *CycE*^{2/+}; *hh*^{bar3}/*hh*^{bar3} flies showed the smaller and blistered phenotypes (Fig. S8A-C). We also observed genetic interaction between *hh* and *cyclin E* in the extra crossvein phenotype. While *CycE*^{JP/+} and *hh*^{2/+} flies showed normal wings, fifty-four percent of *CycE*^{JP/+}; *hh*^{2/+} flies exhibited wings with the extra crossvein (Fig. S8D-F). Collectively, these data suggest that Apt controls wing development by inducing appropriate amounts of Hh and Cyclin E.

230

231 DISCUSSION

Here, we revealed that the transcription factor Apt regulates *Drosophila* wing development, at least in part, through directly activating the expression of *hh* and *cyclin E* to control wing patterning and growth. Both loss-of-function and gain-of function assays clearly demonstrated that Apt is vital for wing development. Further studies showed that knockdown of *apt* attenuated, while overexpression of *apt* activated the expression of *hh* and *cyclin E*.

In the wing disc, Hh exclusively expresses in the P compartment. After many modifications, the mature Hh ligands are secreted from the P compartment and reach ~12cell rows near A/P boundary of the A compartment (Basler and Struhl, 1994; Tabata et al., 1992; Tabata and

Kornberg, 1994). *Ci* expresses solely in the A compartment (Slusarski et al., 1995). Without the Hh, full-length *Ci* is ubiquitinated by SCF^{Slimb} to partial degradation, culminating in formation a truncated transcriptional repressor termed *Ci^R*. *Ci^R* enters into the nucleus to repress the expression of *hh* in the A compartment (Aza-Blanc et al., 1997; Jiang and Struhl, 1998; Smelkinson and Kalderon, 2006). In this study, we found the ubiquitous expression of Apt in the wing disc (Fig. 1A,B). However, the expression of *hh* is restricted in the P compartment of DP cells. Overexpression of *apt* in the wing disc with the MS1096-GAL4 driver emerges the ectopic expression of *hh* in the A compartment, suggesting that Apt is sufficient to turn on *hh* expression. We speculate that during the normal development progress, Apt might cooperates with others factors (such as *Ci^R*) to restrict the expression region of *hh*. It is interesting to investigate the relationship between *Ci^R* and Apt.

To assess the importance of the Apt-binding site in the promoter region of *hh*, we first tried a transgenic reporter assay. However, the regulatory region of *hh* encompassing the upstream region and the 1st intron (~15 kb) (Lee et al., 1992) is too large to make a reporter construct for conventional P-element mediated transgenesis. Therefore, we employed the CRISPR-Cas9 system (Kondo and Ueda, 2013) to mutagenize the endogenous Apt-binding site in the *hh* promoter. All 6 independent mutants exhibited the same phenotypes (reduced expression of *hh*, reduced wing size and the space between L3 and L4), suggesting that the observed phenotypes are not due to off-target effect of Cas9. Nevertheless, we inspected the possibility of off-target effect. Since our gRNA carries the binding sequence for Apt, a binding site of Apt in other than the *hh* promoter could be the most likely candidate for off-target. However, all the 6 mutants showed the wild type sequence around the Apt-binding site in the *cyclin E* promoter (Fig. S9). Furthermore, transheterozygotes between *hh²* and *hh^{AptDB1}* exhibited the *hh* mutant phenotype, smaller wing with extra crossvein. Taken together, these data strongly suggest that the observed phenotypes are not due to off-target effect.

Although our data strongly support that Apt is a transcription factor of *hh*, mutating the Apt binding site on *hh* promoter does not induce severe phenotypes. Beside Apt, other factors might also regulate *hh* transcription. Therefore, both knockdown and overexpression of *apt* only moderately affect the expression of *hh*. Hh, an important morphogen, plays multifaceted roles in segmentation and wing patterning. Previous findings paid more attention on the

protein modification of Hh. The mechanism underlying *hh* transcription is not clear. Here our studies unveil that Apt acts as a transcription factor of *hh*.

While Hh has been implicated in induction of Cyclin E through Ci (Duman-Scheel et al., 2002), subsequent researches have shown that Cyclin E accumulates in the *Mad^{l-2}Su(H)ci* mutant cells (Firth and Baker, 2005). So whether Hh activates *cyclin E* is controversial. In this study we showed that RNAi-mediated knockdown of *hh* or ectopic expression of *hh* in the anterior compartment did not change the expression of *cyclin E*. Taken together, these observations argue against the notion that *hh* regulates *cyclin E* in the wing disc.

Hyperactivation of Hh pathway has been complicated in many tumors (Clement et al., 2007; Jiang and Hui, 2008). It will be fruitful to investigate whether Apt is upregulated in Hh-related tumors. The previous work indicates that Apt involves in tumorigenesis (Woodhouse et al., 2003). It is also interesting to explore whether Apt regulates tumorigenesis through activating Hh signaling. Our finding that Apt regulates wing development through activating *hh* raises a possibility that Apt acts as a potential clinical target for Hh-related tumors.

MATERIALS AND METHODS

Drosophila Strains

Strains used were as follows. *apt^{P44}* (Eulenberg and Schuh, 1997), *apt^{p2}* (Liu et al., 2003), *cycEPlacZ* and *cycEMPLacZ* (Liu et al., 2014), *UAS-apt* (gift of D. Montell), *UAS-GFP* (gift of Y. Hiromi), *hh^{Mir}* was obtained from *Drosophila* Genetic Resource Center. *hh²*, *hh^{Mrt}*, *hh^{bar3}*, *CycE²*, *CycE^{IP}*, *hh-LacZ*, *MS1096-GAL4*, *sd-GAL4*, *dpp-Gal4*, *ptc-GAL4* and *UAS-hh^{RNAi}* were obtained from Bloomington *Drosophila* Stock Center. *UAS-apt^{RNAi}* was obtained from Tsinghua Fly Center. *y²cho²v¹*; *attP40{nos-Cas9/CyO, y¹ v¹ P{nos-phiC31\int.NLS}X}*; *attP40, y² cho² v¹, y² cho² v¹/Y^{hs-hid}*; *Sp/CyO, y² cho² v¹*; *PrDr/TM6C* were obtained from NIG-Fly.

Clonal analysis

Homozygous *apt* loss-of-function clones were generated by *hs-FLP/FRT* recombination (Theodosiou and Xu, 1998). *FRT42D* and *apt^{P44}/CyO* were recombined to generate *FRT42D, apt^{P44}*. Six pairs of *FRT42D, apt^{P44}* cross to *Gla/CyO* were allowed to lay eggs in

300 G418-containing medium, and then test each line with *apt^{P2}/CyO. hs-FLP; FRT42D*,
 301 *Ubi-GFP/CyO* crossed with *FRT42D, apt^{P44}/CyO* were performed at 25°C. Heat shocks were
 302 performed 32-56 hours after egg-laying for 1.5 hours at 37.5°C.

303

304 **Generation of CRISPR constructs**

305 To induce mutations in the Apt-binding site in the *hh* promoter region, we used a Cas9–gRNA
 306 system. We designed gRNA in the *hh* promoter region carrying the binding sequence of Apt
 307 (Fig. 3A). The corresponding sequence was introduced into the pBFv-U6.2 vector and the
 308 gRNA transgenic flies were generated as described (Kondo and Ueda, 2013). gRNA females
 309 were crossed to Cas9 males to obtain the founder animals. Male founders were crossed to
 310 female balancer. Offspring male flies were balanced and stocked. Genomic DNA was
 311 extracted from each offspring male and used for molecular characterization. PCR primers
 312 were designed to construct gRNA expression vectors and to amplify the promoter region of
 313 *cyclin E* (Table S1).

314

315 **RT-qPCR analysis**

316 Wing discs were dissected from 40 third instar larvae. Total RNA was prepared from the
 317 dissected tissues using an RNAprep Pure Tissue kit (TIANGEN #DP431). cDNAs were
 318 synthesized using a Prime ScriptTM II 1st strand cDNA synthesis kit (TaKaRa #6210A). qPCR
 319 was conducted with Bio-Rad CFX96 real-time system using a SuperReal PreMix Plus (SYBR
 320 Green) Kit (TIANGEN #FP205) in a 20 ul reaction containing 2 pmol of relevant primers.
 321 The amount of mRNA was normalized to that of control tubulin mRNA. PCR primers were
 322 designed to amplify the *hh* region (Table S1).

323

324 **Antibodies and Immunohistochemistry**

325 Staining of larval tissues was performed as described previously (Liu et al., 2014). Larvae
 326 were dissected in PBS, fixed in 25 mM PIPES-KOH (pH 7.0), 0.5 mM EDTA, 0.25 mM
 327 MgSO₄ and 4% formaldehyde for 40 minutes on ice and then permeabilized for 15 minutes at
 328 room temperature in PBS containing 0.5% NP-40. The following primary antibodies were

used in overnight incubations at 4°C in blocking solution: rabbit anti-Apt (1:1000) (Liu et al., 2014), rabbit anti-Hh (1:800, gift of T. Tabata), rabbit anti-GFP (1:200, Molecular Probes), mouse anti-GFP (1: 400, Molecular Probes), rabbit anti-β-galactosidase (1:2000, Cappel), rabbit Caspase3 (1:50, Cell Signaling Technology), mouse anti-β-galactosidase (1:500, Sigma), FITC-conjugated phalloidin (1:200, Sigma), mouse anti-Ubx (1:10, Developmental Studies Hybridoma Bank (DSHB)), goat anti-Cyclin E (1:200, Santa Cruz). The secondary antibodies used were as follows: Alexa 488 donkey anti-rabbit IgG conjugate (1:500, Molecular Probes), Alexa 488 donkey anti-mouse IgG (1:500, Molecular Probes), Cy3-conjugated donkey anti-mouse IgG (1:500, Sigma), Cy3-conjugated goat anti-rabbit IgG (1:500, CWBIO), bovine anti-goat IgG-CFL 555 (1:500, Santa Cruz). Mounting used VECTASHIELD Mounting Medium with DAPI (Vector Labs). The caspase-3 staining was did as described previously (Rudrapatna et al., 2013).

341

342 **Microscopy and Image Treatment**

Images were acquired in Leica TCS SP5 confocal microscope and Olympus cellSens, treated with Adobe Photoshop CS6 image programs. Wing size and space between vein 3 and vein 4 or that between vein 1 and vein 2 were measured on each picture using the ImageJ computer program.

347

348 **Statistical analysis**

Results are given as means SEM; each experiment included at least three independent samples and was repeated at least three times. Group comparisons were made by two-tailed unpaired Student's t-tests. *P < 0.05; **P < 0.01, and ***P < 0.001.

352

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357

358 **Competing interests**

359 The authors declare no competing financial interests.

360

361 **Author Contributions**

362 X.F.W., S.H., and Q.X.L. designed research, X.F.W., Q.C., and C.L.F. performed experiments
363 and X.F.W., Z.Z.Z., S.H., and Q.X.L. analyzed data and wrote the manuscript.

364

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368

369 **Supplementary information**

370 Supplementary information available online at

371 <http://dev.biologists.org/lookup/suppl>

372

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492

Figure legends

Fig. 1. APT is expressed in the wing disc and required for wing development.

(A) A single optical section of the PE of the wing disc from a second instar larva and the co-localization of APT and Ubx (PE marker). (B) A single optical section of the DP of the wing disc from a second instar larva. (C) The adult wing of *yw*. (D) The adult wing of *apt^{PJ4}* clones. The arrow indicates the blistered wing. (E) The adult wings of *sd-GAL4; UAS-GFP* and *sd-GAL4; UAS-apt^{RNAi}*. (F) Quantification of the intervein region between L3 and L4 relative to that between L1 and L2 (*sd>GFP* value was set as 100%) of *sd-GAL4; UAS-GFP* and *sd-GAL4; UAS-apt^{RNAi}* by ImageJ. Error bars, SEM. Student's t tests, ***p < 0.001. (G) The adult wing of *MS1096-GAL4; UAS-apt*. (H) The adult wing of *sd-GAL4; UAS-apt*. Scale bars, 200 μ m.

504

Fig. 2. APT regulates the expression of hh.

(A-C) Genetic interaction between *apt* and *hh*. Adult wings of *apt^{PJ4}/+; +/+* (A) and *+/+; hh²/+* (B) show normal pattern. (C) Forty percent of *apt^{PJ4}/+; hh²/+* wings exhibited abnormal morphologies in anterior crossvein (ACV). An arrowhead indicates the extra ACV. Total

509 numbers of analyzed wings were A, 158; B, 157; C, 116. (D-F) The expression of Apt (D) and
 510 *hh-lacZ* (E) in PE cells. (G-I) The expression of Apt (G) and *hh-lacZ* (H) in DP cells. (J-L)
 511 The decreased expression of *hh-lacZ* (K) in the *apt^{PA4}* clones of the PE (J). Clones are marked
 512 by white-dotted lines. (M-O) The decreased expression of *hh-lacZ* (N) in the *apt^{PA4}* clones of
 513 the DP (M). Clones are marked by white-dotted lines. (P-R) Overexpressed Apt (P) increased
 514 the expression of *hh* (Q).

515

516 **Fig. 3. Apt directly regulates the expression of *hh* through its binding site in the *hh***
 517 **promoter region.**

518 (A) Schematic representation of the Apt-binding site in the genomic sequence of *hh*. The
 519 arrow represents transcription start site and the numbers in base pairs are distance from the
 520 start site. (B) Sequences of a wild-type allele and a heterozygous mutant of *hh^{ΔaptDB1}*. The
 521 sequence of the mutant allele was inferred by subtracting a wild-type sequence from the
 522 mixed sequence. The deleted sequence is highlighted in yellow. (C) Cas9-induced
 523 mutagenesis at the *hh* locus. The *hh* locus in Cas9-induced mutants was PCR-amplified and
 524 sequenced. The wild-type sequence is shown at the top as a reference. The Cas9-gRNA target
 525 sequence is underlined with the protospacer-adjacent motifs (PAM) indicated in green.
 526 Deleted nucleotides in *hh^{ΔaptDB1}* are shown as dashes. The deletion size is shown next to the
 527 sequence. (D) RT-qPCR analyses of *hh* mRNA in the wing disc of third instar larvae from *yw*
 528 or *hh^{ΔaptDB1}*. Error bars, SEM from three independent experiments. Student's t tests, **p* < 0.05.
 529 (E) The wing disc of third instar larvae from *yw* or *hh^{ΔaptDB1}* was stained with an anti-Hh
 530 antibody. The expression levels of Hh were determined by mean fluorescence. Error bars,
 531 SEM. ****p* < 0.001. (F-I) Deletion of the Apt-binding site in the *hh* promoter affects wing
 532 development. The wing size and the intervein region between L3 and L4 relative to that
 533 between L1 and L2 (control value was set as 100%) were decreased in *hh^{ΔaptDB1}*. Error bars,
 534 SEM. Student's t tests, ****p* < 0.001. *hh²/+* (G) or *hh^{ΔaptDB1}/+* (H) adult wing shows a normal
 535 phenotype. All adult wings of *hh^{ΔaptDB1}/hh²* transheterozygotes exhibited abnormal
 536 morphologies in ACV (I). An arrowhead indicates the extra ACV. Total numbers of analyzed
 537 wings were G, 157; H, 132; I, 74. Scale bars, 200 μm.

538

539 **Fig. 4. Apt controls the expression of cyclin E.**

540 (A-C) The expression of Apt (A) and Cyclin E (B) in the PE of control wing disc. (D-F)
 541 Decreased Cyclin E (E) expression in the *apt* mutant clones (D). (G-I) Overexpressed Apt
 542 with MS1096-GAL4 (G) increased the expression of Cyclin E (H). (J, K) The expression of
 543 Cyclin E in the wing disc from the *hh^{RNAi}* knockdown (J) and *hh* gain of function mutant
 544 animals (K). (L) Regulation of *hh* and *cyclin E* by Apt in the wing disc. Wing discs from third
 545 instar larvae of wild-type (A-C), *hs-FLP; FRT42D, Ubi-GFP/FRT42D, apt^{P44}* (D-F),
 546 *MS1096-GAL4; UAS-apt* (G-I), *MS1096-GAL4; UAS-hh^{RNAi}* (J) and *hh^{Mrt}* (K) animals were
 547 stained with the anti-Apt antibody (A, G), the anti-GFP antibody (D), the anti-Cyclin E
 548 antibody (B, E, H, J and K). (C, F, I) Merged images of A and B, D and E, G and H,
 549 respectively.

551 **Fig. 5. Apt directly regulates the expression of cyclin E through its binding site in the**
 552 **cyclin E promoter region.**

553 (A) Schematic illustration of the lacZ reporter construct driven by the *cyclin E* promoter
 554 carrying the wild type Apt binding site. (B-D) The reporter *cycEPlacZ* (C) was coexpressed
 555 with the endogenous Apt (B) in the wing disc. (E) Schematic illustration of the lacZ reporter
 556 construct driven by the *cyclin E* promoter carrying the mutant Apt binding site. (F-H) The
 557 expression of reporter *cycEMPlacZ* (G). Wing discs of third instar larvae were stained with
 558 anti-Apt antibody (B and F) and anti-β-galactosidase antibody (C and G). (D and H) Merged
 559 images of B and C and of F and G, respectively.

561 **Fig. 6. Apt is required for production of proper cell number and arrangement of wing**
 562 **discs.**

563 (A-C) Cell number (B, marked by DAPI) and array of cells (C, marked with phalloidin) were
 564 affected in an *apt* mutant clone (A, lack of the GFP signals and marked with a broken line).
 565 (D, E) Cell number (D) and array (E) from a wild-type wing disc were visualized with DAPI
 566 (D) and phalloidin (E). (F, G) Overexpression of Apt in the wing pouch resulted in slight
 567 decrease in the cell number (F) and irregular arrangement of cells (G). (H-J) Apoptosis was
 568 barely detectable in the *apt* mutant clones (H, I) and wild-type wing disc (J). (K)

569 Overexpressed Apt in the wing disc increased the number of apoptotic cells. The wing discs
 570 of *hs-FLP; FRT42D, Ubi-GFP/FRT42D, apt^{PA4}* (A-C, H, I), wild-type (D, E and J) and
 571 *MS1096-GAL4; UAS-apt* (F, G and K) animals were stained with the anti-GFP antibody (A
 572 and H), DAPI (B, D and F), phalloidin (C, E and G) and Caspase-3 antibody (H-K).

573

A **B** **C** **D** **E** **F** **G** **H**

Figure 1

Figure 2

Figure 4

A **B** **C** **D** **E** **F** **G** **H**

Figure 5

Figure 6